

STUDIES ON PREPARATIVE POLYACRYLAMIDE
DISC ELECTROPHORESIS

by

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INTRODUCTION

Analytical electrophoretic techniques have been the subject of many improvements and developments after their introduction early in the twentieth century. These techniques developed from Tiselius' moving-boundary electrophoretic apparatus (92)(93) to the more recent development of disc electrophoresis (22)(62)(63). Preparative electrophoretic methods, however, have not generally attained the degree of sophistication or wide application obtained with analytical methods. Better preparative separation techniques are needed to accelerate and simplify the isolation and purification of proteins and enzymes. This paper deals with a model preparative electrophoretic system which illustrates a practical approach to the one step purification of a single component from a large quantity of complex mixture.

Literature Review

Electrophoresis, General Considerations.

Electrophoresis is the migration of charged particles through a supporting medium under the influence of an electrical field. Techniques in electrophoresis have undergone a series of changes since electrophoretic migration was first observed in the early nineteenth century by the Russian physicist Alexander Reuss (81). One of the earliest electrophoretic techniques developed was a microscopic method, in which the migration of particles could be observed in a glass tube placed on the microscope stage (1). This method was quite useful in the study of surface changes on biological particles. A farther reaching development was the moving-boundary method, in which displacement

of a boundary rather than of individual particles was observed (54). The basic apparatus and technique introduced by Tiselius (92)(93) readily lent itself to the study of proteins and other charged particles of high molecular weight. Tiselius' electrophoretic apparatus served as a basis from which many improvements developed. These improvements made electrophoresis an increasingly important and useful biochemical tool especially for the characterization, isolation and purification of proteins and enzymes.

Zone electrophoresis is a more recent technique, in which separation is accomplished in a liquid medium supported on porous support, rather than in an unimpaired column of liquid (13)(84)(88). Such systems give separations essentially the same as Tiselius' familiar schlieren patterns, but the zones are observed after staining the support to expose separated bands. Smithies (85) described a much improved zone electrophoresis in starch-gel. This support consists of thin slabs of partially hydrolyzed starch gel in an appropriate buffer system. Protein mixtures separate in starch gel on the basis of charge, but much greater resolution is obtained than on other supports because a "sieving" action of the gel structure also selects molecules on the basis of molecular size. When this technique was applied in two dimensions, more than 30 protein components could be demonstrated in human serum.

Raymond and co-workers (75)(76)(77) first reported polyacrylamide gel as a suitable medium for zone electrophoresis. They showed this gel to be completely insoluble, stable against temperature or pH changes, and compatible with many buffer systems. Ease of handling and gel matrix reproducibility, which resulted in improved protein patterns, were cited as further justification for the use of acrylamide

gel in place of starch-gel. Controllable pore size, which is accomplished by varying acrylamide monomer concentration, and more uniform pore structure facilitated a better protein separation with molecular sieving.

The most recently developed electrophoretic technique--disc electrophoresis--was introduced by Leonard Ornstein and Barush Davis (22) (62)(63). This technique is only a few years old, however, James Kendall (45) as early as 1923 first used Kohlrausch's "regulating function" (48) to establish the "steady-state stacking" phenomenon which is a unique feature of disc electrophoresis. Using agar-agar gel columns, Kendall was able to separate a number of rare earth ions from one another (44)(46). This work remained obscure in the literature until 1953, when Longworth (55) reviewed Kendall's work and demonstrated the separation of dissociated salts which involved steady-state stacking. In 1957 Poulik (69) observed that a discontinuous buffer system in starch gel could appreciably improve the resolution of some proteins.

In developing disc electrophoresis, Ornstein and Davis (63) took advantage of the Kohlrausch "regulating function" by establishing pH discontinuities in cylindrical columns of polyacrylamide gel. Such columns are now widely used in the analytical separation of proteins and other charged macromolecules. In this technique electrophoresis is performed in acrylamide gel columns consisting of three sections: (a) sample gel, (b) spacer gel, and (c) running gel (see Fig. 1, p. 4). The running gel consists of smaller pore gel (7% acrylamide) where the proteins are separated electrophoretically according to their mobility and by molecular sieving according to their size. The name "disc"

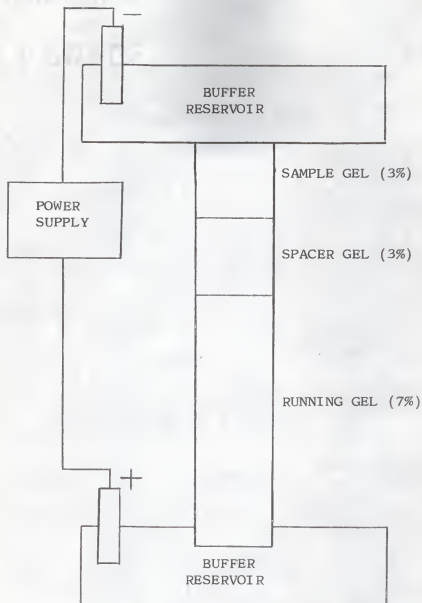


Fig. 1. Disc electrophoresis is performed in a column of polyacrylamide gel consisting of three sections, along with electrode reservoirs, electrodes and a power supply.

electrophoresis was derived primarily from discontinuities in the buffer pH between sample and running gel. It has also been associated with the discoid shape of the separated protein bands.

Development of Preparative Electrophoretic Techniques.

Earlier types of preparative electrophoresis apparatus were based on: (a) differences in isoelectric points of the components being separated, (b) mobility differences, and (c) the phenomenon of electrodecentration and electrophoretic convection.

Foster and Schmidt (30) first introduced an apparatus based on the isoelectric points as the criteria for protein separation. Other modifications of their apparatus were tried by various investigators (3)(6)(21)(31)(32)(35)(82); Williams and Waterman (95) expanded the theory and application to multichambered cells. More recently Bier (9)(10)(11) introduced a forced-flow electrophoresis apparatus based on this technique, which separated isoelectric protein from migrating protein.

Because buffered solutions are often required with biological systems, separation by mobility differences, rather than by isoelectric differences, is more important. Theorell (89)(90)(91) and Hahn and Tiselius (38) have described an apparatus based on this concept. In 1938 Tiselius (94) enlarged his analytical moving-boundary system to a preparative apparatus. Various modifications were tried to improve the technique (7)(12)(13)(26)(49)(50)(65)(86).

Polson (65) and his collaborators introduced the phenomenon of electrodecentration to protein separation, and Kirkwood (47) introduced electrophoresis-convection, a combination of electrophoresis and thermal diffusion, to separate proteins. Other workers (16)(18)(51)(52)(65)

(66) have used and expanded these techniques.

Considerable preparative work is done with zone electrophoresis in vertical columns or gel slabs. Coolidge (20) first introduced a preparative zone electrophoresis method in vertical columns containing ground glass wool. Perhaps the most successful large-scale preparative apparatus was developed by Porath (68), in which separation was obtained in large cellulose powder columns. Most current preparative zone column methods involve cellulose (27)(33)(67)(68), starch granules (8)(25)(26)(28)(41)(42), agarose (39)(40), or plastic powders (36)(60)(61). Starch-gel (34)(83)(85) and agarose (5)(70)(79) gel slabs instead of vertical columns have been tried in preparative zone electrophoresis methods.

Since the introduction of disc electrophoresis, there have been attempts to utilize it for preparative work in vertical columns. Lewis and Clark (53) in 1963 described a preparative procedure in which polyacrylamide gel was used as the stabilizing medium for continuous elution. Polyacrylamide continuous elution apparatus introduced by Racusen and Calvanico (71), Jovin et al. (43), and Radhakrishnamurthy et al. (73) were quite effective for fractionation of from 10 to 20 mg protein samples. Their methods differed primarily in the design of elution chamber. Duesberg and Rueckert (24) described an apparatus in which the support medium was polyacrylamide gel in 8M urea. Their apparatus was useful in the purification of proteins soluble in urea or non-ionic detergents. Other preparative polyacrylamide systems have been reported (2)(14)(56)(80).

Gel slab preparative disc procedures have also been tried. Radhakrishnamurthy (72) cut small strips from a polyacrylamide gel slab

(6" x 12") which were stained and background destained to locate protein bands. The gel was sectioned horizontally and the material was isolated by extraction with buffer in a tissue grinder, centrifugation, and dialysis. Raymond (74), fractionated the sample on a vertical polyacrylamide gel slab and then electrophoretically eluted the zones horizontally through the thickness of the gel into a number of micro electro-convection cells.

The majority of the preparative methods reported, both zone and disc, depend primarily on separation of the particles in a support medium, followed by a continuous elution technique to collect the fractionated sample. Ornstein (62) and Jovin et al. (43) have suggested utilizing the "steady-state stacking" phenomenon for large-scale preparative separations. Such an application, although clearly a useful one, has not been reported. The present work is directed toward such an application.

Theoretical Considerations

Polyacrylamide gel has been very successful as an electrophoretic stabilizing medium (22)(77) in the disc electrophoresis method. The gel is essentially a three-dimensional network of interlaced molecules cross-linked at intervals by means of methylene bridges (see Appendix B, p. 62). The effective gel pore size is uniform and is easily controlled by adjustment of the acrylamide concentration. At high acrylamide concentrations (about 7%), the gel serves as a sieving matrix through which the sample is fractionated. At lower concentrations (about 3%), the polyacrylamide gel serves as an anticonvection (spacer gel) medium which allows for "pre-concentration" of the sample into a

thin starting zone. This thin starting zone is responsible for much of the success and uniqueness of the "disc" method. High resolution separations cannot be obtained without thin starting zones.

The theoretical basis for the formation of a suitably thin zone was first established by Kohlrausch (42) in 1897 with the introduction of his "regulating function". This function establishes a set of conditions under which a boundary will be sharply maintained between two ionic species of the same charge, but differing mobility, as they migrate in an electrical field. The less dense and less mobile ion must be located above the more dense and more mobile ion, and the ions must move downward when the electrical field is applied. Concentrations at the moving-boundary other than those specified by the regulating function are automatically regulated and maintained regardless of the initial concentrations or densities provided sufficient time is allowed and convection is prevented.

When proteins or any ions form such a moving-boundary, the velocities of boundary components have to be equal. This condition can be satisfied only if mobility-voltage gradient products are equal.

$$V_1 M_1 = V_2 M_2$$

Thus, when an electrical potential is applied, the leading ion moves down and away from the trailing ion. This leaves behind a region of decreased conductivity and produces an increased voltage gradient in that region. The higher voltage gradient then accelerates the trailing ion, forcing it to catch up with the leading ion. The trailing ion can not go faster than the leading ion; therefore, a steady-state is established. In a more complex system which contains protein molecules of intermediate mobilities, a boundary moves down the column, overtaking

the proteins. Thus the protein molecules stack into thin discs, each in a concentration established by the "regulating function", arranged in order of decreasing mobility and lying between the leading and trailing ions. This "stack" of materials then continues to migrate at a constant rate.

A form of Kohlrausch's function applied to disc electrophoresis by Ornstein (62) may be expressed as

$$\frac{R}{S} = \frac{X_S C_R}{X_R C_S} = \frac{m_R Z_S (m_S - m_T)}{m_S Z_R (m_R - m_T)}$$

This equation gives the ratio of the total concentrations (R and S) of the ionic solutions (r and s) required to initiate and maintain a stable moving-boundary. (See Appendix E, p. 64, for derivation of the above equation from first principles.) The fraction of dissociation is x, c the concentration, m the mobility, z the ionic charge, and r, s, and t are ions, with t of opposite charge and common to both solutions.

In disc electrophoresis this "regulating function" governs the concentration of sample protein in each segregated band. Under given pH conditions, the mobility of the leading ion, s, and the trailing ion, r, can be adjusted to that the mobilities of a mixture of protein molecules (of the same charge as ions r and s) are of intermediate values between r and s. For example in serum systems, glycine at pH 8.3 is a good trailing ion with an effective mobility, $m_r x_r$, of -0.5 units (1 mobility unit equals 10^{-5} cm²/volt-sec). Chloride is the leading ion with a pH independent mobility of -37 units. Since most serum proteins have mobilities between -0.6 units and -7.5 units at pH 8.3, concentrations of these protein molecules will automatically be regulated as described.

In analytical applications the stacked bands enter a second region of gel and encounter a discontinuity of pH set to be near the pK_2 of the trailing ion. This causes the mobility of the trailing ion to become almost that of the leading ion and certainly faster than the fastest protein molecule. For example, the glycine trailing ion has a mobility of 0.5 units at pH 8.3 and a mobility of -5.0 units at pH 9.5. The mobility of the fastest protein remains almost constant over this pH region. The non-protein boundary rapidly moves ahead of the proteins. These discs of stacked protein molecules are thus left in a uniform voltage gradient, where they are separated as in ordinary zone electrophoresis.

Statement of Problem

The most common approach to preparative electrophoresis at present remains scale-up of analytical systems with either continuous elution from columns or "batch" elution from gel segments. These techniques require long running times for elution of the slower moving components from the column or slab. Also, the need for relatively large volumes of elution buffer in continuous systems causes recovered fractions to be quite dilute in most cases. These methods have led to a number of successful protein separations, but seldom on a large scale. A workable large-scale preparative electrophoretic method would therefore be an important addition to the methodology for isolation and purification of proteins and enzymes.

Ornstein (62) mentioned steady-state stacking as an approach toward large-scale preparative disc electrophoresis method and stated that:

"If very large quantities of protein are used...and are gelled in place on top of another very long column of, for example, regular spacer gel, then if a current is passed when the protein front has migrated sufficiently far, all the protein will have stacked; but now the individual elongated discs will have thicknesses about equal in centimeters to the percentage of the individual proteins in the serum... Except for the individual concentration gradients across the boundaries between two discs, the elongated discs will contain pure proteins in high concentration and can, for example, be 'electrophoresis' out of the lower end of the column into a properly designed fraction collector."

Joven et al. (43) also have referred to the possibility of using steady-state stacking for preparative work and stated that their "apparatus may also be adaptable for preparative procedures in which large amounts of material are stacked on a single length of suitable upper gel and eluted in order of component mobilities without separation between successive bands." Although both Ornstein and Jovin specifically mentioned the possibility of using large-scale stacking, no reports incorporating such suggestions have been published.

The present work therefore concerns a system to achieve stacking conditions in a simple and convenient large-scale preparative polyacrylamide gel procedure. Such a procedure would be quite useful in the purification of a single component from a large quantity of complex sample. However, the principle of the steady-state stacking method precludes electrophoretic separation of the stacked proteins. The stacked bands must therefore be separated mechanically.

In the present investigation, cholinesterase was separated from horse (equine) serum. Cholinesterase is found in horse serum in appreciable amounts, is a relatively stable enzyme, and is easy to determine quantitatively. These properties make it an attractive model component. Cholinesterase was formerly known as "serum cholinesterase" or "pseudocholinesterase," but more recently it has been designated

"cholinesterase" (3.1.1.8) (23) and should be distinguished from "acetylcholinesterase" (3.1.1.7) (23), the predominant acetylcholine hydrolyzing enzyme of nervous tissue and the erythrocytes. Carboxylesterase (3.1.1.1) (23), another esterase present in horse serum (4), served as the component from which cholinesterase was separated.

An important aspect of disc electrophoresis methodology is the staining of protein bands after electrophoresis. To evaluate preparative procedures, many analytical runs must be made and stained for protein. Amido Black, the dye most widely employed for protein staining in polyacrylamide gels (22), requires an electrophoretic destaining step. Because of this rather awkward and time consuming step, the use of a more rapid protein dye method was investigated.

Fazekas de St. Groth et al. (87) illustrated the use of Coomassie Brilliant Blue R250 (also designated acid blue 83 $\overline{\text{CI 42660}}$ in the Color Index) to measure protein on cellulose-acetate strips. He found that Coomassie Blue followed Beer's law between 1 and 10 μg protein applied across 1 cm width of cellulose acetate strip. Protein staining on filter paper, agar and starch gel are also possible with Coomassie Blue; however, acrylamide gel was not tried. Therefore, the use of a Coomassie Blue method as a useful substitute for Amido Black staining of polyacrylamide gels was investigated. Results are reported in this paper.

MATERIALS AND METHODS

Materials for Polyacrylamide Disc Electrophoresis

Apparatus. (See Figs. 2 and 3, p. 14).

Buffer Reservoirs. Round polyethylene refrigerator dishes (94 mm diameter x 55 mm deep) were used for buffer reservoirs in the analytical procedure. Six 3/8 inch holes were drilled (equidistant and on a 30 mm radius) in the bottom of the upper reservoir. Rubber electrical grommets (1/4" i.d., 3/8" mtg. hole) were inserted into the holes to position the electrophoresis tubes. The upper reservoir used for the preparative apparatus was similarly constructed, but contained four 5/8" holes. Rubber serum bottle stoppers with 6 mm holes cut through served as grommets for the preparative system. These specially made grommets were cemented in place with white silicon rubber (Silastic Bathtub Caulk, Dow Corning).

Electrophoresis Electrodes. Graphite electrodes from flashlight batteries (8 mm x 60 mm) or cylindrical pieces of rigid plastic (9 mm x 60 mm) wrapped with about 10 turns of platinum wire were used as electrodes. They were held in place in the center of the buffer reservoirs (upper and lower of both analytical and preparative apparatus) by hollow rubber stoppers (B-D "Vacutainer" stoppers, 6 mm i.d. and 7 mm deep) cemented in the center of the dish with silicone rubber caulk.

Electrophoresis Tubes. Tubes used for analytical runs were cut from pyrex tubing (5 mm i.d. and about 80 mm in length); the ends were lightly fire polished. Cellulose nitrate centrifuge tubes (10 mm diameter x 80 mm and 16 mm diameter x 100 mm) served as sample tubes in preparative electrophoresis runs.



Fig. 2. Analytical disc electrophoresis apparatus.



Fig. 3. Preparative disc electrophoresis apparatus.

Solutions.

Reagents. Acrylamide monomer, N,N'-methlenebisacrylamide (BIS) and N,N,N',N''-tetramethylethylenediamine (TEMED) were obtained from Eastman Kodak Company; ammonium persulfate, sulfosalicylic acid and glycine from Fisher Scientific; tris(hydroxymethyl)-aminomethane (TRIS) from Matheson Coleman and Bell; and riboflavin from Merck Sharp and Dohme. All were reagent grade and were used in both analytical and preparative polyacrylamide gels without further purification.

Sample Source. Horse sera were obtained through courtesy of the Dykstra Hospital staff, College of Veterinary Medicine, Kansas State University.

Stock and Working Solutions. The stock and working solutions used in polyacrylamide gel formation were those employed by Davis (22), except working solutions No. 3 and No. 4, which were prepared according to Table I and Table II, page 16.

Polyacrylamide Disc Electrophoresis Procedure

Analytical Procedure.

The procedure used for analytical polyacrylamide gel electrophoresis was essentially that of Davis (22), with certain modifications found useful in our application.

The electrophoresis tubes were first thoroughly brushed and rinsed with detergent, then they were rinsed with 1% Kodak Photo-Flo solution and allowed to dry. The last step proved important for easy gel removal. A mounting board for holding tubes while the gel polymerized contained 5/8" holes drilled 3/16" deep to hold inverted B-D Vacutainer test tube stoppers. These stoppers had just the right size cavity to

TABLE I. Stock Solutions

Reagent A		Reagent B		Reagent C	
1 N HCl	48 ml	1 N HCl	48 ml	Acrylamide	28.0 g
TRIS	36.6 g	TRIS	5.98 g	BIS	0.735 g
TEMED	0.23 ml	TEMED	0.46 ml	water to	100 ml
water to	100 ml	water to	100 ml		
(pH 8.9)		(pH 6.7)			

Reagent D		Reagent E		Reagent F	
Acrylamide	10.0 g	Riboflavin	4 mg	Sucrose	40 g
BIS	2.5 g	water to	100 ml	water to	100 ml
water to	100 ml				

Reagent G		Reagent H	
Ammonium		TRIS	6.0 g
Persulfate	0.14 g	Glycine	28.8 g
water to	100 ml	water to	1000 ml
		(pH 8.3)	

TABLE II. Working Solutions

	Parts	Reagent		Parts	Reagent
No. 1	1	A	No. 3	1	B
Small-Pore	2	C	Large-Pore	2	D
(7% acrylamide)	1	water	(3% acrylamide)	1	water
	4	G		4	G

No. 2	1	B	No. 4	2	B
Large-Pore	2	D	Large-Pore	3	D
(3% acrylamide)	1	E	(2% acrylamide)	3	F
	4	F		8	G

support a gel tube vertically and prevent the contents from leaking out.

The small-pore gelling solution was prepared according to Table II, No. 1, page 16. Wintrobe type pipettes (diSPo pipettes, Scientific Products) were used to add the gel solution to about 20 mm from the top. Water was carefully layered over the gelling solution to form a flat gel followed by careful layering with water to produce a flat interface. Mixing of the water and the gel solution must be avoided. The small-pore solution polymerizes after 30 to 40 minutes. The water layer is then removed, the tube is rinsed with a small amount of the large-pore solution, and approximately 0.15 ml of a large-pore solution (Table II, No.2, p. 16) is added to form the spacer gel. Again, it is necessary to overlay the polymerizing gel solution with water to form smooth, flat gel surfaces. The spacer gel is polymerized by irradiation with a 15 watt daylight fluorescent lamp for 25 to 30 minutes. In No. 3 large-pore solution (Table II, p. 16), ammonium persulfate was used as the polymerizing catalyst rather than riboflavin. This reagent was an adaptation in these experiments which made observation of fluorescent protein bands in the gels possible. Gelling agents containing riboflavin interfered with such observations.

After removal of water above the spacer gel, sample was added to the electrophoresis tube. Horse serum (0.03 ml) was mixed with 1.5 ml of large-pore solution, and 0.15 ml of the mixture was added to each tube. This corresponds to 3 μ l of serum per tube (about 250 μ g of protein). Once layered over the spacer gel, the sample solution was photopolymerized as before, except that it was not necessary to layer the solution with water. If samples were mixed with No. 3 large-pore solution, however, layering with water was required to provide anaerobic

conditions for the persulfate catalyzed polymerization. Quite often it was found more convenient to mix horse serum with 40% sucrose stabilizing medium instead of the large-pore gelling solution. A second polymerization step was thus eliminated. This had no apparent effect on the electrophoretic separation. Careful layering of tray buffer over the sucrose sample was required, however.

After the gel tubes were ready for electrophoresis, they were carefully removed from the rubber stopper supports. (Tight fitting tubes experience suction which may disrupt the gels). The tubes then were fitted into the upper reservoir grommets, and a 1/10 strength stock buffer (Reagent H, Table I, p. 16) was used to fill both upper and lower reservoirs. The upper reservoir with electrophoresis tubes protruding downward was positioned over the lower reservoir to make contact with the buffer, and was oriented so the vertical tubes were each equidistant from the central electrode.

A Buchler Instruments Model No. 3-1014A Regulated D. C. Power Supply was employed in the constant current mode for all electrophoresis experiments. The cathode was attached to the upper reservoir and the anode to the lower one. Generally, 5 to 6 mA per tube was employed; however, with 40% sucrose as the sample diluent, about 2 mA per tube was required for an initial 5 minutes, after which the current per tube could be raised to 5 or 6 mA. This procedure was required to prevent loss of sample into the upper reservoir by convection. Electrophoresis was continued for 30 to 40 minutes. Occasionally a trace of Bromphenol Blue was added to the upper reservoir; it migrated near the moving-boundary in the small-pore gel and therefore served as a visual marker of the front.

At the completion of electrophoresis, the reservoir buffers were poured off, tubes were removed from grommets, and gels were removed from the glass electrophoresis tubes by rimming with a 19 gauge needle and water. The needle was introduced between the gel and glass tubing from the top with a short vibrating motion while water was added with a wash bottle to lubricate the gel surface. The vibrating motion with the needle was continued while the gel tube was rotated until the gel slipped out. Properly cleaned electrophoresis tubes were essential to obtain undamaged gels.

Gels then were fixed and stained with Amido Black according to Davis (22), or by a method to be described later (p. 21). In either case the gels were stored for observation in small tubes containing 7% acetic acid.

Preparative Procedure.

Cellulose nitrate centrifuge tubes with bottoms sliced off were used for electrophoresis tubes. It was not necessary to clean or rinse these tubes with Kodak Photo-Flo, because there was very little adhesion between gel and tube. For this reason the bottom of the tube was not removed until just prior to electrophoresis. The tubes were held upright by placement in rubber serum stoppers.

The first gel layer was a 10-15 mm small-pore plug at the bottom. The solution was layered with water and allowed to polymerize for 50 to 60 minutes. Because of the somewhat poor adhesion between gel and plastic tube, longer polymerizing times and more careful handling was necessary.

After polymerization of the small-pore plug and removal of the water layer, a 2% acrylamide large-pore solution (Table II, No. 4, p. 16)

was prepared and added to the tube to about 30 mm from the top. The solution was layered with water and allowed to polymerize for 75 to 90 minutes until a slightly opaque whitish gel was obtained. The sample was then added after removal of the water layer.

Sample was prepared by addition of one part Reagent B (Table I, p. 16) to eight parts of horse serum. Thus 1 ml of serum and 0.125 ml of Reagent B were mixed and added to the tube. The remainder of the tube was filled with reservoir buffer. In these experiments the sample solution was dense enough to allow over-layering of reservoir buffer, although sucrose could have been added to the sample if required.

The tubes were carefully fitted into place in the upper reservoir, and buffer was added over the sample with a pipette until the upper reservoir level was reached. About 300 ml of reservoir buffer was then added to both reservoirs.

The gel tubes were ready for electrophoresis except for removal of bottom sections to allow electrical contact between reservoirs. The tip of a short 3 mm diameter pyrex tube was heated and touched to the bottom of the gel tube so that a small piece of cellulose nitrate melted away. Care was taken to melt the opening slow enough to avoid disruption of the gel by heat or mechanical force. This left a neat, circular opening in the bottom of the tube to provide electrical contact, but a small rim remained for mechanical support of the tube contents. This support is essential to the success of the method, for if there is a break between tube wall and gel, the sample will migrate preferentially down the side and separation of layered discs is impossible. In the extreme case when a rim support is not provided the gel will simply slip out during the run.

Electrophoresis then was performed at a constant current of 1 mA per tube initially for one hour to allow the moving-boundary and the "regulating function" to take effect. The current was increased to 3-3.5 mA per tube for an additional 3-3.5 hours, after which current was reduced to 1-1.5 mA per tube for an additional 1-1.5 hours. After electrophoresis, the tubes were removed from the reservoir and frozen by immersion in liquid nitrogen or crushed dry ice.

Polyacrylamide Gel Bands Staining Procedures

Protein Staining

Amido Black. Some gels in this study were stained with Amido Black according to Davis (22). The method is briefly described as follows. A 1% combination stain and fixation solution is prepared with 1 g dye dissolved in 100 ml 7% acetic acid. Upon use of dye in acetic acid solution, proteins are fixed and stained in the gel in one process. The gels are stained for at least 1 hour, then rinsed with running tap water. Background stain is removed electrophoretically at 15 mA per tube for 15 to 20 minutes. The same apparatus for analytical electrophoresis is also used for destaining, except that destaining tubes are slightly larger and constricted on one end to facilitate reinsertion and holding of gels.

Coomassie Brilliant Blue R250. The electrophoretic destaining of Amido Black was found awkward and time consuming, so another method was sought. Coomassie Brilliant Blue R250 (Imperial Chemical Industries, Organics Inc.) was investigated as a successor to Amido Black for disc electrophoresis protein staining, based on the work of Fazekas de St. Groth et al. (87). St. Groth had shown Coomassie Blue readily applic-

able to protein staining on cellulose acetate strips. Coomassie Blue was recently employed by Meyer and Lamberts (58) for the detection of microgram quantities of parotid saliva proteins on acrylamide-gel strips. Their staining procedure consisted of staining gel strips in 0.25% Coomassie Blue (methanol-glacial acetic acid-water solvent, 5:1:5, v/v) for 20 minutes. They, too, removed the background stain electrophoretically, so their method offers little advantage over Amido Black. In 1966 Maizel (57) reported use of Coomassie Brilliant Blue R250 to stain radioactive type-2 adenovirus proteins fractioned in polyacrylamide gels. His staining procedure consisted of an 18 hour fixation in 20% sulfosalicylic acid followed by 3 hours staining in 0.25% Coomassie Blue. The background dye in this case was removed with six 20 ml washings of 7% acetic acid.

A simpler Coomassie Blue method, devised for protein staining in the work reported here, consists of placing gels in 20% aqueous sulfosalicylic acid for 20 minutes to fix the protein bands, followed by staining in 0.25% Coomassie Brilliant Blue R250 for 2 hours. The background dye is readily removed by washing with tap water. The gels are placed in 7% acetic acid for storage.

Enzyme Staining.

β -Carbonaphthoxycholine Iodide Enzyme Stain. Esterase enzyme bands can be located in acrylamide gels by staining with chromogenic substrate reagent. Ravin *et al.* (78) demonstrated the specificity of β -carbonaphthoxycholine iodide for cholinesterase (3.1.1.8) in a colorimetric estimation, as well as in a histochemical demonstration and localization procedure. This dye was used in a similar manner to detect the presence of cholinesterase band in the polyacrylamide gel

after electrophoretic fractionation. The staining solution contained 20 mg β -carbonaphthoxycholine iodide (Nutritional Biochemicals Corp.), 20 mg diazo blue B (du Pont Naphthanil Diazo Blue B; Nutritional Biochemicals Corp.), 1.5 ml of 2% CaCl_2 , and 20 ml of 0.1 M pH 7.4 phosphate buffer in a final volume of 100 ml. The combined reagent is unstable and must be made fresh before each use. The gel was immersed in staining solution for 20 to 30 minutes, after which it was rinsed with tap water and placed in a small test tube containing 7% acetic acid for storage. An insoluble red azo dye was deposited where cholinesterase had cleaved the substrate. (See Appendix C, p. 62, for the reaction of the enzyme with β -carbonaphthoxycholine iodide and subsequent coupling.)

Indoxyl Acetate Fluorescent Substrate and Stain. Guilbault and Kramer (37) introduced indoxyl acetate as a fluoregenic substrate for the determination of cholinesterase. In the present work it was found to be a useful companion substrate to β -carbonaphthoxycholine iodide for locating gel esterase bands. A 0.108 M stock solution of indoxyl acetate (K and K Laboratories, Inc.) in dioxane was stored at 5°C and a working substrate solution was prepared as needed by addition of 0.1 ml of stock solution to 3.9 ml of 0.1 M pH 6.5 phosphate buffer. Electrophoresed gels in tightly stoppered tubes of substrate-buffer solution were placed in a U. V. viewer (Chromato-Vue Model C-3, Ultra-Violet Products, Inc.), and the fluorescent bands were observed in long wavelength U. V. light.

After 30 to 45 minutes, the fluorescent bands disappeared and a stable blue-green stain appeared in their place. These substrate stained bands were readily observable in room light and were superimposed on the position of the fluorescent bands, although because of

diffusion they were not as sharp as the original fluorescent bands. The colored product is derived from its fluorescent precursor according to the reaction scheme of Guilbault and Kramer (37). (See Appendix D, p. 63).

Protein Assay

The Miller modification (59) of the Folin-Lowry Method (29) was used as the protein assay procedure. The method is briefly outlined below; slight changes have been introduced.

Reagents.

Folin Phenol Reagent. To one hundred g $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$, 25 g $\text{Na}_2\text{MO}_4 \cdot 2\text{H}_2\text{O}$ and 700 ml water were added to 50 ml 85% H_3PO_4 and 100 ml concentrated HCl. The mixture was refluxed gently for 10 hours, after which 150 g Li_2SO_4 , 50 ml water and a few drops of liquid bromine were added. The mixture was again boiled for 15 minutes without a condenser to remove excess bromine. It was cooled, diluted to 1000 ml and filtered through a sintered glass funnel. For use this solution is diluted 1:11.

Alkaline Copper Reagent. Solution A was prepared by dissolving 0.2 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 0.6 g of tris(hydroxymethyl)-aminomethane in 100 ml of distilled water.¹ Solution B was prepared by mixing 10% Na_2CO_3 and 0.5 N NaOH. The working alkaline copper reagent was prepared by mixing 1 part Solution A with 10 parts Solution B. This mixture had to be prepared fresh every 2 to 3 days. Solution A and Solution B are stable.

¹This alternate to the usual tartrate reagents is an unpublished method developed in this laboratory by B. A. Cunningham, D. D. Louie, and L. M. Nicholson.

Assay Procedure.

One ml of protein solution and 1.0 ml alkaline copper reagent are mixed, allowed to stand at room temperature 10 minutes, and then 3.0 ml diluted molybdate reagent is added to each sample. The samples are then incubated at 50°C for 10 minutes, cooled, and read at 650 mμ on a Coleman Junior Spectrophotometer (Model 6).

Enzyme Assay

The cholinesterase method of Guilbault and Kramer (37) previously described for gel staining, was employed with slight modification to quantitatively determine enzyme eluted from gels. The rate of indoxyl acetate cleavage was measured fluorometrically on a Model 110 Turner Fluorometer (G. K. Turner, Associates, exciting wave length 395 mμ and emission wavelength 470 mμ). The assay procedure employs a small aliquot of enzyme added to 0.1 M pH 6.5 phosphate buffered substrate brought to a constant temperature of 30°C in small pyrex tubes (12 mm x 75 mm). The fluorometer dial reading is adjusted to zero after indoxyl acetate substrate solution (prepared as on p. 23) is added to the assay tube. To start the reaction, 0.1 ml of enzyme solution is rapidly added and mixed with the indoxyl acetate substrate solution. The change in fluorescence was observed over a 2 minute period. One unit of enzyme activity is defined as that amount causing a change of one fluorescent unit in a 1 minute reaction time ($\Delta FU_1 \text{ min}$). The specific activity is defined as one unit of enzyme activity per mg protein.

Stock indoxyl acetate in dioxane spontaneously decomposes to the fluorescent indoxyl compound and this can be detected visually by a

color shift from light violet in fresh stock to light yellow. Because of this situation stock indoxyl acetate solution was made up in only 1 ml volumes and used within 1 hour. Similarly the working substrate solution (stock plus buffer) should be used within 1 hour.

Microtome Slicing of Preparative Gels

A microtome-cyrostat¹ (Ames Lab-Tek, Inc.) was used to cut the frozen preparative size polyacrylamide gels after steady-state stacking of horse serum.

Frozen gels were first sliced with a razor blade to isolate the piece containing stacked sample. This piece was then attached to the specimen positioning block by freezing. A frozen conical support around specimens built up with 1% to 2% Jaguar J2S-1 solution (Stein, Hall and Co., Inc.) served to stabilize the gel cylinder during the slicing operation. Jaguar, a water soluble polysaccharide thickener, replaced the usual embedding agents such as Lab-Tek O.C.T. Compound which contain protein. The microtome blade and sample were positioned at least one-half an hour before the slicing operation to insure equilibration at the working temperature of -30°C .

Five 20 micron slices were made for each aliquot down the length of the gel. Each five-slice set was collected on a toothpick and transferred to a sample tube. The toothpick was discarded after each transfer. A series of 100 μ gel sections was collected and suspended in 0.5 ml of 0.1 M pH 6.5 phosphate buffer, covered with Parafilm (Mara-

¹Kindly made available by Dr. G. B. Marion, Department of Dairy and Poultry Science, Kansas State University.

thon) and stored at 4°C overnight or for at least 12 hours. After this time essentially all the elutable material was removed from the thin slices, and analysis could be performed.

RESULTS

Coomassie Brilliant Blue R250 Study

Effect of Sulfosalicylic Acid and Coomassie Blue Combined for One-Step Protein Fixing and Staining.

In an attempt to achieve a one-step staining method, 0.25% Coomassie Brilliant Blue was prepared in 20% sulfosalicylic acid solution. Three μ l of equine serum was electrophoretically separated with 5 mA per tube for 40 minutes by the method described on page 13. Gels were stained for 75 minutes and then rinsed with tap water. This procedure deeply stained the entire gel similar to Amido Black. Attempts to destain these gels in a number of other soaking baths were not completely successful. Electrophoretic destaining (45 minutes at 15 mA per tube) was unsuccessful, for band migration and streaking occurred.

Effect of Different Dye Solvent pH.

Various buffers were next tried as solvent for 0.25% Coomassie Brilliant Blue R250 protein dye, e.g., pH 8 and 9 TRIS buffers, 2% K_2HPO_4 , 2% K_3PO_4 , pH 3 citrate buffer, and deionized water. Three μ l serum samples were electrophoretically separated as before at 5 mA per tube for 40 minutes. The gels were then fixed for 20 minutes in 20% sulfosalicylic acid and stained with 0.25% Coomassie Blue in the various dye-solvent solutions for two hours. The Coomassie Blue in water gave sharpest protein bands. The 2% K_3PO_4 dye solution was least effective of the groups, 2% K_2HPO_4 , TRIS buffers, and citrate buffer all

allowed reasonable staining, but the protein bands were more diffuse than those from the water-dye solution.

Effect of Various Fixers on Gel Protein Band Staining.

Three μ l serum samples were electrophoretically separated as before at 5 mA per tube for 40 minutes. Ten minute fixing with 20% sulfosalicylic acid was used for a control gel. Twenty minute fixing times were then tried in 7% acetic acid, 10% trichloroacetic acid (TCA), 10% formalin and 20% sulfosalicylic acid all followed by a two hour staining period in 0.25% Coomassie Brilliant Blue R250. Twenty minutes in 20% sulfosalicylic acid was found to be the best fixer for protein in the polyacrylamide gels, for this leaves protein bands somewhat darker than the 10 minute control, without excessive gel background staining. Judged by the number and sharpness of bands resolved after 0.25% Coomassie Blue staining, 20% sulfosalicylic acid was superior to 7% acetic acid, 10% TCA or 10% formalin.

Effect of Staining Time on Various Volumes of Horse Serum.

Five sets of serum samples (0.1, 0.5, 1.0, 2.0 and 3.0 μ l) were analytically separated in 7% polyacrylamide running gel, 3% spacer gel and a 40% sucrose sample section. Electrophoresis was at 1 mA per tube for 5 minutes followed by 40 minutes at 6 mA per tube. After fixation in 20% sulfosalicylic acid for 20 minutes, gels representing each set of five serum volumes were stained with 0.25% Coomassie Brilliant Blue R250 for $\frac{1}{2}$ hr., 1 hr., 2 hrs., 4 hrs., and overnight (14-18 hrs.). The results are shown in Fig. 4, p. 29.

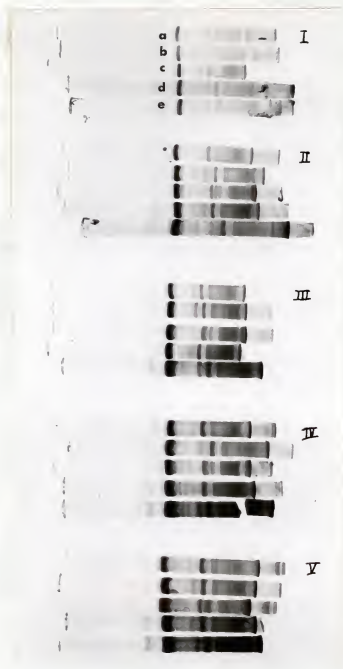


Fig. 4. Effect of protein concentration and Coomassie Blue stain time on band resolution. Protein expressed as volume of horse serum: I) 0.1 μ l, II) 0.5 μ l, III) 1.0 μ l, IV) 2.0 μ l, and V) 3.0 μ l. Stain times: a) $\frac{1}{2}$ hr., b) 1 hr., c) 2 hrs., d) 4 hrs., and e) overnight.

Model Preparative Polyacrylamide Gel Study

Standard Protein Curve.

Bovine serum albumin (Armour Pharmaceutical) was used as the standard to prepare a protein curve. Approximately 3.5 mg of bovine serum albumin was dissolved in 10 ml of water and the concentration determined from the 1%, 1 cm extinction of 6.6 at 280 m μ (19). Using suitable dilutions and a modified Folin-Lowry protein assay procedure (59), a curve, linear to 0.60 absorbance (0.12 mg protein), was obtained as shown in Fig. 5 below.

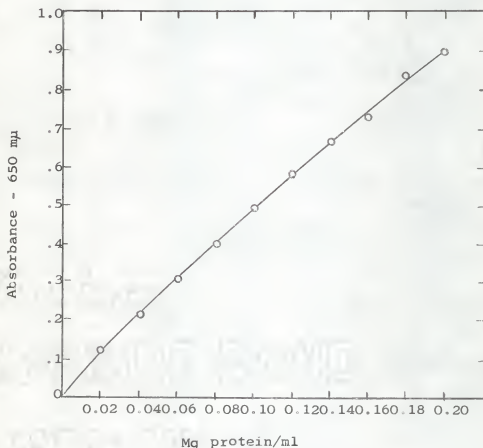


Fig. 5. Standard Protein Curve

Sensitivity of Fluorescent Assay Procedure for Cholinesterase Activity.

To determine the response of the enzyme assay in detecting small changes in cholinesterase, the change in fluorescent units (Δ FU) per minute was determined with horse serum used as the enzyme at 0.1 μ l increments up to 2.0 μ l. As Fig. 6, p. 32 indicates, the FU was found to be linear over the 3 minute reaction period for each dilution. A plot of enzyme activity expressed as Δ FU/min. vs. serum volume showed the assay method to be applicable for enzyme concentration determinations (Fig. 7, p. 32), even at the lowest level employed in analytical separations.

Establishment of the Model System.

Horse serum samples were fractionated analytically to identify components which could be studied in a model preparative procedure. The sample gels were formed from No. 1 small-pore running gel¹, No. 3 large-pore spacer gel and 40% sucrose-serum sample area containing 3 μ l of serum. They were electrophoretically fractionated with 2 mA per tube for 5 minutes followed by 6 mA per tube for 35 minutes. No. 2 large-pore acrylamide was not used, because the fluorescent riboflavin interfered with the subsequent observation of fluorescent bands in the gel. There were no observable differences in the protein pattern when analytically fractionated horse serum samples in No. 2 or No. 3 spacer gels were compared. The first sample was stained with 0.25% Coomassie Blue R250; the second with 1% Amido Black. Both showed a normal horse serum fractionation pattern. The third gel was immersed in β -carbo-

¹For No. 1, No. 2, and No. 3 gel preparations, see Table II, p. 16.

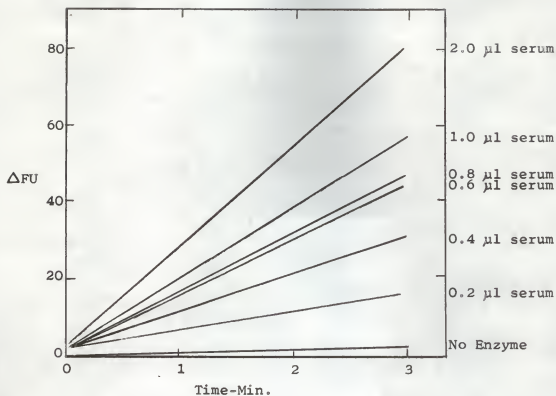


Fig. 6. Cholinesterase Assay. Progress curves at 0.108 M indoxyl acetate which show response at various enzyme levels.

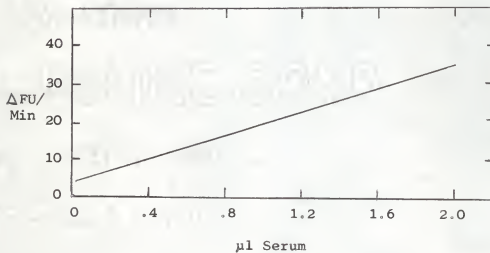


Fig. 7. Cholinesterase Assay. Rate of fluorescence change directly proportional to enzyme concentration.

naphthoxycholine iodide which localized cholinesterase as a red band about 2 to 3 mm down into the small-pore (Fig. 8, p. 34). A fourth and fifth gel, immersed in indoxyl acetate solution and viewed under U. V. illumination, showed two very distinct fluorescent bands. After about 30 minutes, the fluorescence disappeared and visible blue bands formed in its place. One band was 2 to 3 mm and the other 12 to 13 mm down into the small-pore gel as shown in Fig. 8, p. 34. Separated horse serum plus indoxyl acetate thus produced two components which could be made visible either by their initial fluorescence or by their permanent blue coloration. This method was then employed to judge the effectiveness of separation in the preparative procedure.

Protein Stacking in 3% Polyacrylamide Gel.

Analytical electrophoresis tubes (6 mm pyrex) were used in which a small amount of 7% acrylamide gel was used as a bottom plug. About one-half of the remaining space was filled with 3% polyacrylamide spacer or stacking gel, and 0.5 ml of sample was placed over this. The sample gel was prepared with a polymerizing solution prepared from one part large-pore buffer solution (Reagent B)¹, two parts small-pore acrylamide solution (Reagent C), one part water and four parts 0.14% ammonium persulfate. The polymerizing solution was mixed with an equal volume of diluted horse serum in 40% sucrose mixture to give serum equivalents of 0.025 ml, 0.05 ml, 0.10 ml, 0.15 ml, 0.20 ml, and 0.25 ml when the one-half ml was polymerized over the stacking gel. The resulting sample gel concentration was about 3½% acrylamide. The serum load was about 10, 20, 40, 60, 80, and 100 times the amount normally used in an

¹ For Reagent B and C preparation, see Table I, p. 16.



Fig. 8. Analytically fractionated horse serum (3 μ l) stained with: a) β -carbonaphthoxycholeline iodide; b) indoxyl acetate; and c) 0.25% Coomassie Blue.

analytical run. All samples were run simultaneously and for 60 minutes (smaller samples) or 80 minutes (the three larger samples) at 2 mA per tube. The 3% polyacrylamide gel used to form the stacking area does not form rigid gels, so the samples were somewhat difficult to remove from the electrophoresis tubes. After careful removal, they were placed in indoxyl acetate solution, and position of the fluorescent bands was observed under U. V. light.

The first two tubes, which contained 10 and 20 times of the amount of sample normally used in an analytical run, had two distinct fluorescent bands in the stacking area. The remainder of the tubes had one band remaining in the sample area plus one in the stacking area. These last tubes should have been run longer. The fluorescent bands increased in width as the serum sample increased. The first tube had

very thin sharp bands about 1 mm apart which had moved about 5 mm into the stacking gel. In the second tube the bands were about 2 or 3 mm apart and again moved about 5 mm into the stacking gel. In the remainder of the samples, the faster moving fluorescent component moved from 5 to 8 mm into the spacer gel, while the slower moving fluorescent band had not moved out of the sample gel. The component which remained in the sample area could still be observed as a band, although it was not as distinct as the one in the stacking area.

Suitable Stacking Gel Medium.

Seakein Agarose (Bausch and Lomb) and Jaguar J2S-1 (Stein, Hall and Co., Inc.) were investigated as possible substitutes for polyacrylamide in the stacking gel area of the preparative method. Concentrations of 0.25%, 0.5%, and 1% agarose in water and Reagent B¹ (7:1, v/v) were tried for the stacking medium. A sample layer containing 0.15 ml of horse serum was prepared with horse serum, 40% sucrose and Reagent B (4:3:1, v/v). The tubes were run for 1 hour at 1 and 2 mA per tube. The sample did concentrate but no protein bands could be seen to indicate stacking of the protein. No fluorescent banding was observed in the 1% agarose gel after immersing in indoxyl acetate solution. The whole area where the sample was located in the gel seemed to fluoresce without banding.

A 1% Jaguar J2S-1 solution in 40% sucrose, water and Reagent B (4:3:1, v/v) was also tried as the stacking medium. The sample solution was identical to that used in the agarose trial. The tubes were run at 1 mA per tube for one hour and 2 mA per tube for one hour. This poly-

¹For Reagent B preparation, see Table I, p. 16.

saccharide thickener was quite difficult to work with because a flat interface between the sample layer and stacking layer could not readily be formed and there was usually mixing of these two layers while electrophoresis was being carried out. The sample was concentrated but no banding occurred.

Various concentrations of acrylamide gel were then investigated to find one most suitable for stacking. With 0.15 ml horse serum in the sample gel, a 2% acrylamide spacer and a 3% acrylamide spacer gel were tried at 2 mA per tube for 80 minutes. These stacking gels showed very good layering of the proteins in marked contrast to agarose and Jaguar. Although 2% acrylamide gel was very effective as a support medium in the electrophoresis tube, it was quite difficult if not impossible to remove from the tube.

Selection of Sliceable Preparative Electrophoresis Tube.

An electrophoresis tube which could be sliced without first removing the gel would prove very useful, so a number of plastic and paper straws were investigated for this purpose. Stacked protein areas could not be directly observed in paper straws and gels would not adhere to the walls of plastic straws. Even when gels remained in the plastic straws, they were difficult to handle without inadvertently slipping out. Various attempts to plug the end of the plastic straws with glass wool-gel mixtures were unsuccessful, for even these plugs readily fell out.

Cellulose nitrate centrifuge tubes with the bottom removed were then tried. Again the gel did not want to stay in place very well. Various materials such as sodium silicate (water glass) and plexiglass dissolved in methylene dichloride were used to coat the inner wall of

the cellulose nitrate tubes, but such treatment did not appreciably improve adherence of the gel to the tube wall. Finally a workable system was achieved by tube placement followed by partial removal of the tube bottom just prior to electrophoresis (further described on p. 19). The small amount of cellulose nitrate tube remaining as a narrow rim served to hold the gel in place. Electrophoresis was then carried out and results were compared with preparative stacking in pyrex tubing of the same size (10 mm i.d.). Both systems gave equally good results and showed identical stacking much like that in Fig. 9, p. 38.

Further Scale Up of Preparative System.

The size of sample electrophoretically stacked was next extended to 1 and 1.25 ml of horse serum using a 7% gel tube plug and a 2% large-pore spacer gel. The sample, a mixture of eight parts horse serum and one part Reagent B¹, was electrophoretically stacked for 1 hour at 1 mA per tube. The resulting stacked samples are shown in Figs. 9 and 10, p. 38.

The largest volume of horse serum stacked was 4 ml with similarly prepared 16 mm x 100 mm cellulose nitrate tubes. These samples were stacked with 3 mA per tube for 1½ hours, 6 mA per tube for 5½ hours and 3 mA per tube for a final 2 hours. Fig. 11, p. 39, shows a sketch of the proteins in the stacking gel area.

The sample seemed to concentrate first before migrating very far into the stacking gel. A homogeneous yellow area migrated into the stacking gel and after a sufficient time showed the banding as indicated in Figs. 9, p. 38 and 11, p. 39.

¹For Reagent B preparation, see Table I, p. 16.



Fig. 9. Photograph of $1\frac{1}{4}$ ml (a) and 1 ml (b) horse serum preparatively stacked (1 mA/tube for 1 hr., 3 mA/tube for $3\frac{1}{2}$ hrs., and 1 mA/tube for 1 hr.) in cellulose nitrate tubes (10mm x 80mm).



Fig. 10. Photograph of $1\frac{1}{4}$ ml horse serum being stacked in the preparative apparatus.

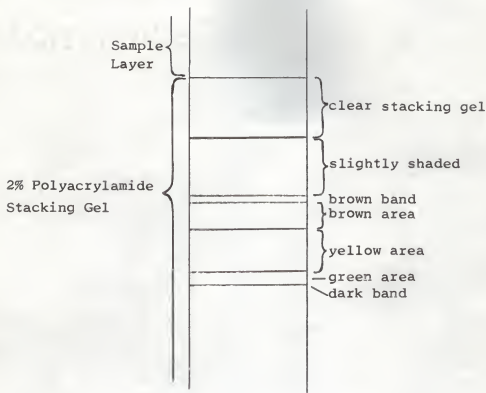


Fig. 11. Sketch of observed banding of 4 ml horse serum after preparative stacking (3 mA/tube for $1\frac{1}{2}$ hrs., 6 mA/tube for $5\frac{1}{2}$ hrs., and 3 mA/tube for 2 hrs.) in a cellulose nitrate tube (16 mm x 100 mm).

Hand Slicing of Stacked Protein Sample.

A 1 ml horse serum sample stacked in 2% spacer gel was frozen and the stacked area cut into thirty slices using a hand held razor blade. Each slice was placed in 0.5 ml of pH 6.5, 0.1 M phosphate buffer for 12 hours and the solution assayed for protein and enzyme activity. The specific activity of the enzyme, defined as the change of fluorescent units per minute per mg of protein, is shown in Fig. 12, p. 40. There was 24% recovery of enzyme activity, and 71% recovery of protein.

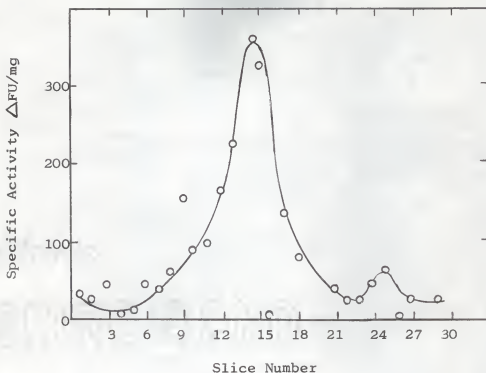


Fig. 12. Specific Activity. Hand sliced horse serum sample (1 ml) preparatively stacked (1 mA/tube for 1 hr., 3 mA/tube for $3\frac{1}{2}$ hrs., and 1 mA/tube for $\frac{1}{2}$ hr.) in 10 mm x 80 mm cellulose nitrate tube.

Microtome Slicing of Stacked Protein Samples.

A tube containing 1.25 ml of serum stacked in a cellulose nitrate tube as previously described was frozen and cut on a microtome. The stacked area was isolated and cut into 138 sections of 100 microns each. Pooled sections were then analyzed after elution. The results are shown for protein distribution (Fig. 13, p. 42), enzyme activity (Fig. 14, p. 43), and specific activity (Fig. 15, p. 44). In this analysis about 40% of the protein and 34% of the enzyme activity was recovered. The active fluorescent areas could be seen in both Figs. 14, p. 43 and 19, 48.

Another sample of 1.25 ml of serum was cut as before into 190 slices of 100 microns. Each sample was assayed for protein (Fig. 16, p. 45) and enzyme activity (Fig. 17, p. 46). The specific activity curve is shown in Fig. 18, p. 47. Protein and enzyme assays were also performed on four broad areas of the gel shown in Fig. 19, p. 48. Table III, p. 48, indicates the distribution of the protein and enzyme found after the stacking. A recovery of 78% of the protein and 70% of the enzyme activity was achieved in this experiment. Again, two fluorescent areas were evident (Figs. 17, p. 46 and 18, p. 47) which showed separation of the two enzymes during stacking.

Composite Mixtures of Sliced Sample Solutions.

Eighteen composite mixtures were prepared by mixing 0.2 ml of each individual sample as indicated in Table IV, p. 49. Three samples of each composite mixture were then analytically fractionated with sample volumes of 0.15 ml. One sample gel was stained with Coomassie Blue, a second with β -carbonaphthoxycholine iodide and the last was stained with indoxyl acetate. The results are shown in Fig. 20, p. 50. Two fluorogenic components are separated by the stacking preparative disc electrophoresis method.

Comparison of Commercial Cholinesterase With Composite Sample.

An analytical fractionation compared composite sample No. 3 with 0.15 mg commercial cholinesterase (3.1.1.8) (Worthington Biochemical) in No. 1 small-pore running gel¹, No. 3 large-pore spacer gel¹ and sample mixed with 40% sucrose. Electrophoresis was carried out for 5 minutes at 2 mA per tube followed by 30 minutes at about 6 mA per

¹For No. 1 and No. 3 gel preparations, see Table II, p. 16.

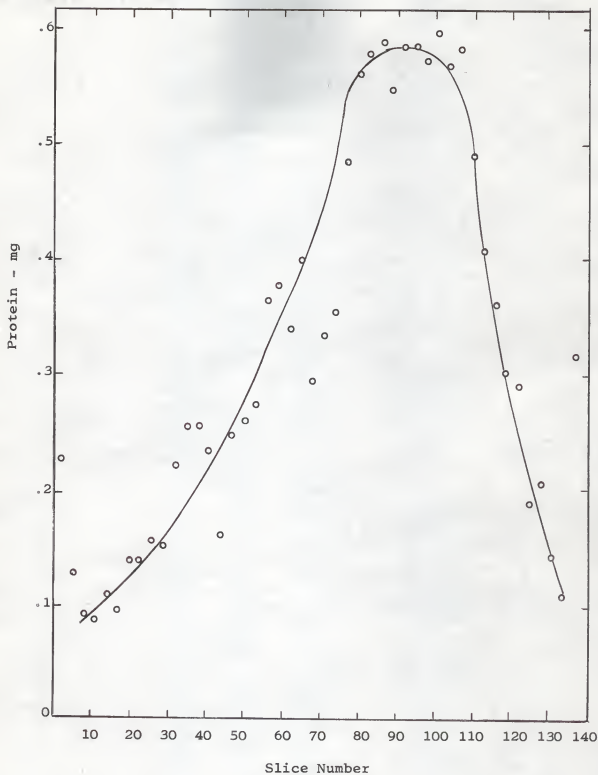


Fig. 13. Graph of protein distribution of first microtome sliced horse serum sample ($1\frac{1}{4}$ ml) after preparative stacking in 2% polyacrylamide gel.

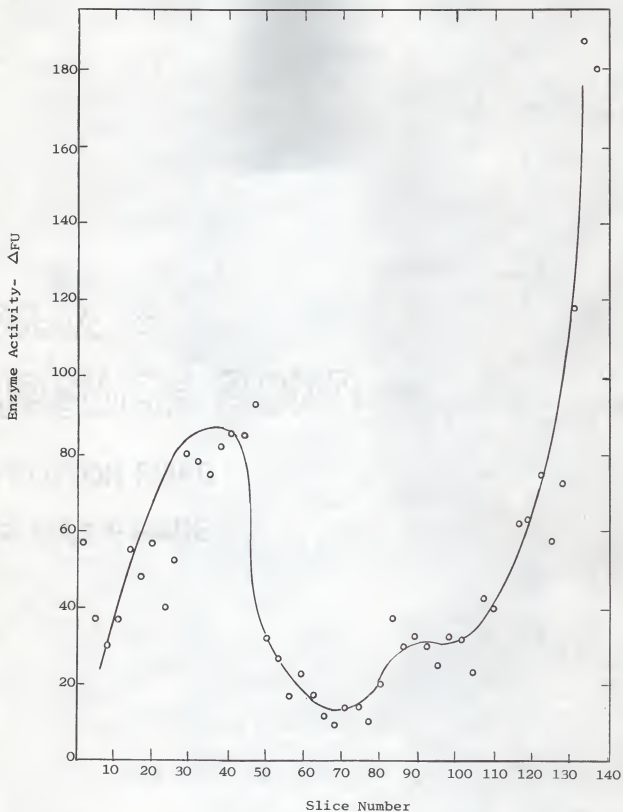


Fig. 14. Graph of enzyme activity distribution of first microtome sliced horse serum sample ($1\frac{1}{4}$ ml) after preparative stacking in 2% polyacrylamide gel.

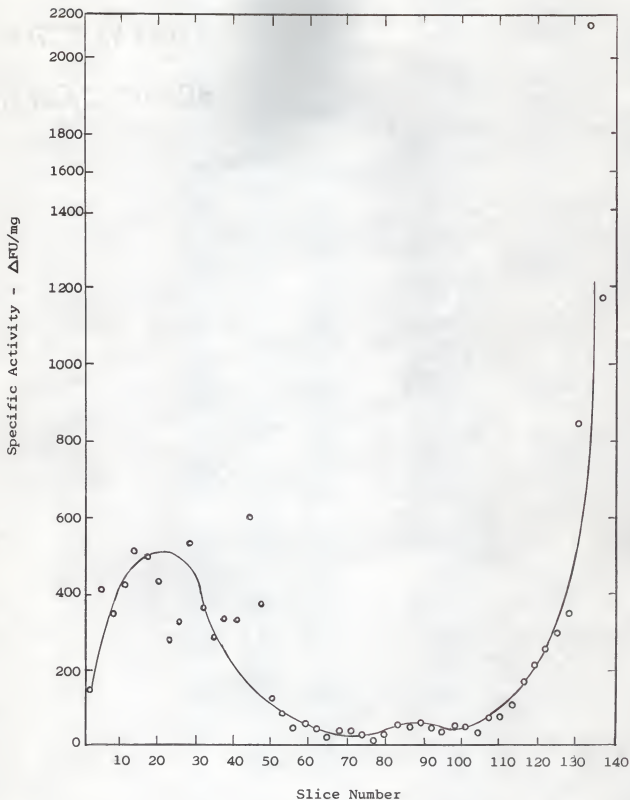


Fig. 15. Graph of specific activity distribution of first microtome sliced horse serum sample ($1\frac{1}{4}$ ml) after preparative stacking in 2% polyacrylamide gel.

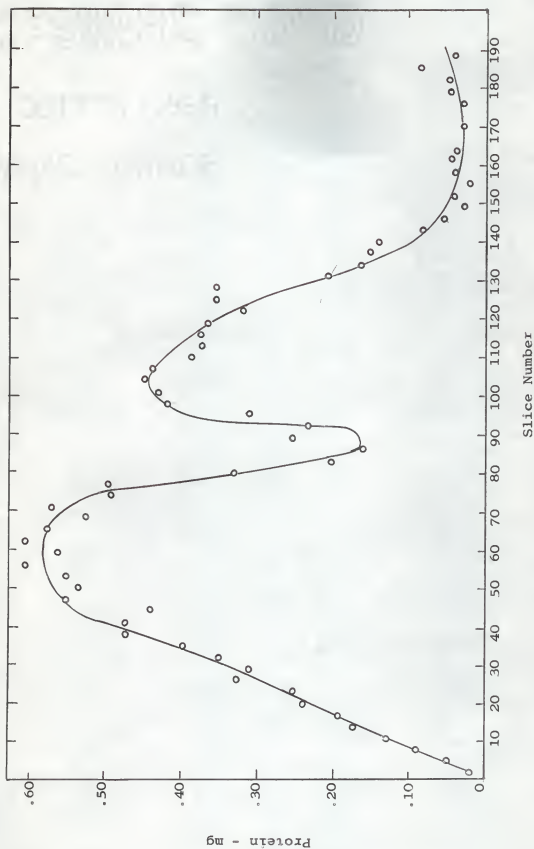


Fig. 16. Graph of protein distribution of second microtome sliced horse serum sample (1 1/2 ml) after preparative stacking in 2% polyacrylamide gel.

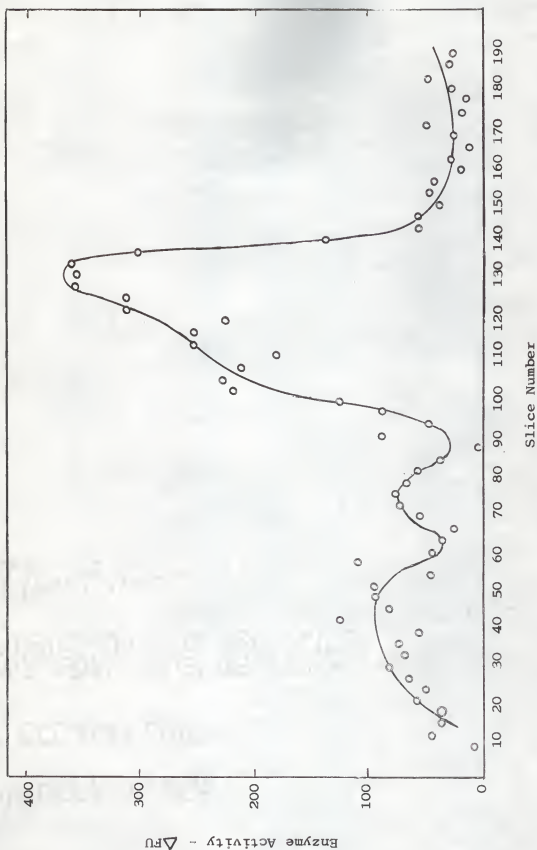


Fig. 17. Graph of enzyme activity distribution of second microtome sliced horse serum sample ($1\frac{1}{4}$ ml) after preparative stacking in 2% polyacrylamide gel.

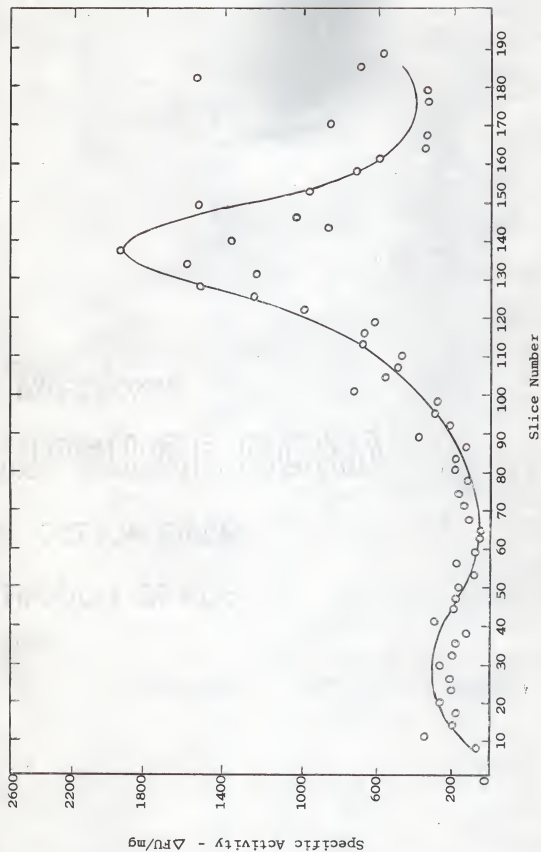


Fig. 18. Graph of specific activity distribution of second microtome sliced horse serum sample (1 $\frac{1}{4}$ ml) after preparative stacking in 2% polyacrylamide gel.

TABLE III. Distribution of protein and enzyme activity in $1\frac{1}{4}$ ml horse serum preparatively stacked.

<hr/>				
<hr/>				
Sample on:	Protein	107.5 mg		
	Enzyme Activity	33.75×10^3	Δ FU/min	
	Specific Activity	0.314×10^3	Δ FU/mg	
Sample off:				
Area (See Fig. 19)	Protein (mg)	Enzyme Activity (Δ FU $\times 10^3$)	Specific Activity (Δ FU/mg $\times 10^3$)	Activity Yield(%)
1	11.5	1.4	0.12	4.1
2	16.4	2.0	0.12	5.9
3	51.0	18.4	0.36	54.6
4	5.1	1.7	0.33	5.0
	84.0 mg	23.5 Δ FU		69.9%
Percentage recovery: Protein 78%				
Enzyme Activity 70%				
<hr/>				

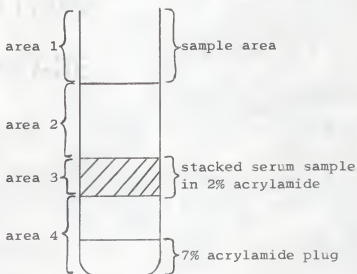


Fig. 19. Assay areas of second preparative stacked horse serum sample ($1\frac{1}{4}$ ml).

TABLE IV. Composite Samples. Distribution of composite sample protein and enzyme levels.

Composite Number	Tubes Included	Protein (mg/ml)	Activity (Δ FU/ml)	Specific Activity (Δ FU/mg $\times 10^3$)	Fold ^{1,2} Purification	Yield ² (%)
1	1- 19	.038	19.8	.52	1.7	1.1
2	20- 30	.096	65.5	.68	2.1	2.1
3	31- 39	.135	65.3	.48	1.5	1.7
4	40- 50	.165	102.0	.62	2.0	3.3
5	51- 59	.188	65.3	.35	1.1	1.7
6	60- 69	.191	39.3	.21	0.6	1.2
7	70- 82	.149	67.8	.46	1.4	2.6
8	83- 88	.059	11.0	.19	0.6	0.2
9	89- 98	.101	66.6	.66	2.1	2.0
10	99-110	.143	244.5	1.71	5.4	8.7
11	111-119	.126	232.3	1.84	5.9	6.2
12	120-130	.093	327.6	3.52	11.2	10.7
13	131-140	.053	250.8	4.73	15.1	7.4
14	141-152	.018	51.5	2.86	9.1	1.8
15	153-160	.010	20.2	2.02	6.4	0.5
16	161-170	.012	28.2	2.35	7.5	0.8
17	171-180	.006	15.9	2.65	8.4	0.5
18	181-189	.014	30.3	2.16	6.9	0.8

¹ Original horse serum specific activity was 0.314 Δ FU/mg $\times 10^3$.

² Pool of composites 9 through 14 (carboxylesterase) show results of 7.0 fold purification; 37% yield. The actual carboxylesterase recovery is greater because the fraction of total initial esterase activity due to carboxylesterase is not known. The 37% yield is therefore a minimal value.

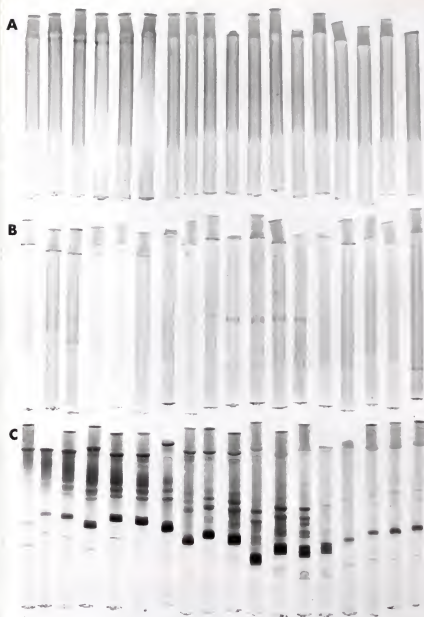


Fig. 20. Series of 18 composite horse serum samples compiled from the second microtome sliced gel after analytical fraction (1 mA/tube for 5 min., 4 mA/tube for 25 min., and 3 mA/tube for 30 min.) and stained with (A) 3-carbonaphthoxycholine iodide, (B) indoxyl acetate, and (C) Coomassie Blue.

tube. One tube each of composite sample and commercial cholinesterase was stained with Coomassie Blue and one of each with β -carbonaphthoxy-choline iodide. Fig. 21, below, shows the results.

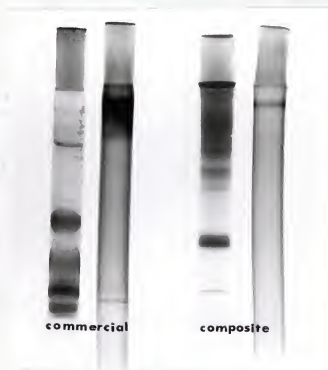


Fig. 21. Analytical disc electrophoresis patterns of commercial cholinesterase (3.1.1.8) and composite sample No. 3, both stained with β -carbonaphthoxycholine iodide and 0.25% Coomassie Blue.

DISCUSSION

De St. Groth et al. reported that in a slightly acidic medium Coomassie Brilliant Blue R250 was bound to NH_3^+ groups of proteins separated and fixed on cellulose acetate strips. Our studies extend the application of such staining to protein bands in polyacrylamide gels and show it to be a very dependable and sensitive dye. The possibility of staining in a one-step process was not realized, however. Coomassie Blue in water will not fix protein bands in the gel, so fixing was accomplished with a 20 minute gel immersion in 20% sulfosalicylic acid. A suitable staining time after protein fixing produced a protein stained gel requiring only a rinse for background destaining. Separation of the fixing and staining steps was necessary because Coomassie Blue in sulfosalicylic acid (one-step approach) produced gels with a deeply stained background. This defeated any advantage of Coomassie Blue staining over Amido Black staining.

Of the various solvents and buffers tried for the Coomassie Blue dye, deionized water performed the best for gels. Since no staining advantage could be seen as a function of pH, deionized water was chosen rather than buffer. After taking gels out of the staining solution and rinsing, a very slight bluish cast remains as background. It does not impair immediate recognition of even the faintest resolved bands, however, and after several changes of a water rinse this bluish cast will have washed out to give a perfectly clear background.

From the results of the various staining times on increasing amounts of serum, it appeared that the larger the amount of sample electrophoretically separated, the shorter the optimum staining time

would be. Fig. 22, below, shows a plot of optimum staining time (judged by the number of bands resolved) as a function of protein applied. A standard fixing time of 20 minutes in 20% sulfosalicylic acid was used. With increased time in the fixer, the amount of dye absorbed by the protein bands in the gel was also increased. Thus by increasing the fixing time, staining times could be shortened. A fixing time of 15 to 20 minutes was found to be the best compromise for fixing the protein bands in acrylamide gels yet obtaining nicely stained bands without too much background.

The above Coomassie Blue staining procedure provided a simple, quick and sensitive method for detecting fractionated protein bands in polyacrylamide gel. Upon storage of the gels in 7% acetic acid, there was no diffusion or fading of the stained protein bands and gels could, therefore, be kept as permanent records of sample fractionations.

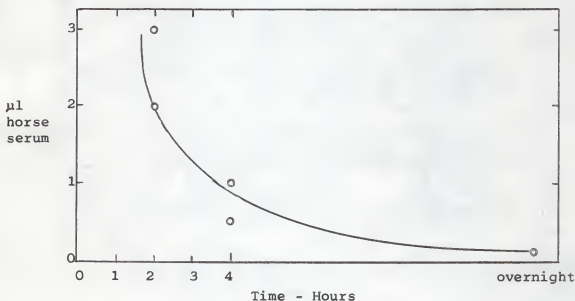


Fig. 22. Plot showing hours of staining required to resolve 12 bands in increasing amounts of horse serum (fixing time of 20 minutes in 20% sulfosalicylic acid).

Horse serum served quite well as a model sample in this study since it was readily available, could be worked with at room temperature, and contained two readily separable and easily detected esterases. After staining with a fluorescent reagent, the esterases provided a visual model for observing the stacking phenomenon and for determining effectiveness of the separation. Augustinsson (4) reported three esterases present in horse serum (arylesterase, aliesterase and cholinesterase) of which aliesterase and cholinesterase actively hydrolyze indoxyl acetate. With β -carbonaphthoxycholine iodide used as substrate, the slower moving component was shown to be cholinesterase (3.1.1.8). Therefore, the faster moving esterase was probably aliesterase. Aliesterase is now known as carboxylesterase (3.1.1.1) (23).

When the stacking procedure was first tried in a 3% polyacrylamide gel, the initial sharpness and distinctness of two fluorescent bands demonstrated quite clearly that the two enzymes had been separated, however sharpness was gradually lost by diffusion. After 30 to 45 minutes, the fluorescence disappeared and visible blue bands formed which were somewhat wider than the initial fluorescent ones.

Although stacking of the protein occurred in a 3% polyacrylamide gel, it seemed to physically break down or distort if the protein moved too fast through the gel. This could be controlled by a reduction of current which resulted in increased electrophoresis time, or, more effectively, by a reduction of gel concentration. A 2% polyacrylamide stacking phase gel was finally chosen because it was the lowest concentration to provide a gel-like matrix.

Banding as a result of steady-state stacking could be seen directly in horse serum samples of 1 ml or greater when they were run pre-

paratively in a 2% polyacrylamide stacking gel. Initially the yellowish serum concentrated to less than one-fourth the original volume with only a slight migration into the stacking gel. It then moved well into the stacking area, became more compact, and formed visible protein zones after electrophoresis for a sufficient length of time. The procedure would seem quite useful in concentrating dilute protein components as well as in stacking proteins preparatively. Larger sample volumes required longer electrophoresis times and longer stacking gel areas.

The 2% gel chosen for optimal stacking was not rigid enough to remove it from tubes but it could be solidified by freezing. Efforts to gently warm the outer tube and extrude the frozen contents were not successful. The use of plastic tubes was therefore developed so that frozen gel and tube could be sliced together and the stacked bands isolated directly. Hand slicing was not precise enough for the sample volume used. A microtome-cryostat, however, proved to be quite suitable for separating the stacked components. With this approach very thin, uniform gel slices could be made while working in a cold atmosphere. Thus, the gel was kept frozen during the slicing procedure and this provided more representative component distribution.

In the first gel sliced with the microtome, good separation of the two esterases was demonstrated although the slicing process stopped somewhat short of the entire fast enzyme band. The slower esterase showed less activity and may have been partially deactivated during the electrophoresis or freezing process or perhaps it was less reactive to indoxyl acetate than the faster esterase. A second stacked sample gel handled similarly showed the same separation of two esterases. In

both cases the peaks of esterase activities were equidistant which showed good reproducibility of the preparative procedure.

The recovery of protein and enzyme activity after electrophoresis was quite good with yields of 78% protein and 70% enzyme activity. Part of the protein loss was probably basic proteins which migrated into the upper reservoir and part of the enzyme activity loss is attributable to deactivation of the slower esterase.

When the serum sample size was increased from 1.25 ml to 4 ml, the stacked protein area showed identical but larger banding. Good protein stacking was obtained even though low current (6 mA per tube) was used. Thus stacking can be obtained with low enough currents to minimize gel temperature rises during electrophoresis. These results indicate the feasibility of scaling steady-state stacking electrophoresis to even larger volumes. The ultimate scale seems limited only by the physical means to support the 2% acrylamide matrix, to remove some heat especially from thicker sections, and to control distortion during freezing.

Composite slice samples which were then analytically electrophoresed served to demonstrate complete separation of the two enzyme components. Comparison of indoxyl acetate stained composite gels (detects both esterases) with β -carbonaphthoxycholine iodide stained composites (detects only the slower esterase) showed the slower esterase distributed over several initial sections while the second esterase was located in several later composites. The banding was therefore not as thin as expected and reduced resolution was no doubt due to some band distortion during both electrophoresis and freezing. A better gel freezing procedure might reduce irregular expansion and improve the

resolution. Although enzyme staining proved separation of two active components, Coomassie Blue stained composites showed that banding into pure protein components was not obtained. Some leakage of the sample between the gel and the tube might have taken place which would account for mixing or protein bands in addition to that caused by band distortion.

Analytical disc electrophoresis comparison of commercial cholinesterase with composite sample No. 3 showed this composite sample to approach the purity seen in a commercial sample of cholinesterase which indicates the potential of this preparative procedure for practical purifications.

From these preliminary results, it seems reasonable to predict that with certain refinements a preparative one-step separation of single component from a mixture based on steady-state stacking of protein in 2% polyacrylamide gel could be achieved. In principle, this procedure could be scaled up to considerably larger volumes with more sample, low current, longer electrophoresis time and longer stacking gel to achieve a layering of proteins in centimeter sized bands. Since it would require many hours for stacking to be completed, temperature labile components could be preserved only by adequately controlling the gel temperature. The use of low currents would minimize temperature gradient effects which could be pronounced in larger tubes. It should finally be mentioned that repeated preparative procedures based on the stacking approach would certainly also be feasible and would greatly enhance the purification. Perhaps two or three passes would suffice to obtain a homogeneous protein from a highly complex initial mixture.

SUMMARY

The first part of this paper describes an investigation of Coomassie Brilliant Blue R250 as a protein stain in acrylamide gels. The staining procedure developed consists of fixing protein bands in the gel with 20% sulfosalicylic acid for 20 minutes followed by staining in 0.25% Coomassie Blue for 2 hours. Background dye is readily removed by a few washes in distilled water. The gels can be stored indefinitely in 7% acetic acid thus affording a permanent record of protein fractionation results. This Coomassie Blue staining procedure eliminates electrophoretic destaining which is required with Amido Black methods and the Meyer and Lamberts (66) Coomassie Blue staining procedure. It provides a very convenient, sensitive method for staining proteins fractionated by polyacrylamide disc electrophoresis.

The second part of this work describes a preparative electrophoresis procedure based on steady-state stacking of the sample components in a 2% polyacrylamide gel. Two esterase components were shown to be present in horse serum with indoxyl acetate as substrate. These enzymes serve as model components to test the effectiveness of the preparative system. The apparatus consisted of cellulose nitrate electrophoresis tubes, a 7% polyacrylamide gel end plug, and 2% polyacrylamide gel as the spacer or stacking gel. After electrophoresis was completed, the steady-state stacked protein layer was frozen, sliced and the slices analyzed for protein and enzyme activity.

The protein, enzyme activity, and specific activity curves showed a broad concentrated area of protein but with two distinctly separated esterases. Analytical fraction by disc electrophoresis of composite

samples made from the individual gel slices was used to further document the separation. Protein was detected with Coomassie Blue and the enzymes determined with indoxyl acetate and β -carbonaphthoxycholine iodide. Comparison of these results showed the two enzyme components to be completely separated. In general, any component can be sliced out of the stacked layer, thus freeing it from much of the remaining mixture. Ideally, it should be possible to obtain homogeneous material in a thin slice from one run. However, at the present stage of development, more than one run would be required to achieve that result.

The size of sample used in the preparative procedure varied from 0.15 ml of serum (13 mg protein) to 4.0 ml of serum (344 mg protein). In every case, the sample was electrophoretically stacked in the 2% polyacrylamide gel. These results suggested that even larger sample volumes probably could be run with increased electrophoresis tube size and increased electrophoresis time.

In conclusion, the author believes this work has illustrated a useful, fairly simple, inexpensive and rapid model for preparative disc electrophoresis. It demonstrated that appreciable initial purification of a single component is possible from a relatively large volume of a complex mixture.

APPENDICES

APPENDIX A

Structure of Materials

1. acrylamide



2. N,N'-methylenebisacrylamide (BIS)



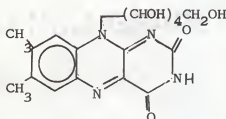
3. ammonium persulfate



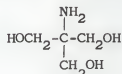
4. N,N,N',N'-tetramethylethylenediamine



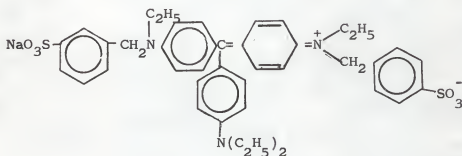
5. riboflavin



6. tris(hydroxymethyl)aminomethane (TRIS)



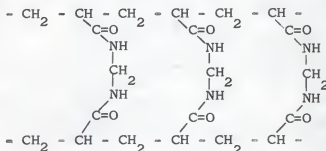
7. Coomassie Brilliant Blue R250



APPENDIX B

Gel Formation

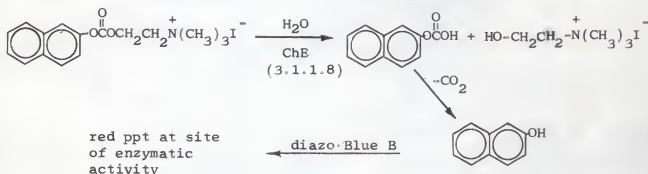
The chemical structure of the gel is a linear polymer of acrylamide, crosslinked by means of methylene bridges. This structure, extended in three dimensions, consists of long hydrophilic chains. There are no hydrophobic chains. There are no hydroxyl or acidic groups except perhaps on the end. Upon polymerization, the acrylamide forms a transparent, flexible, stable, insoluble gel.



APPENDIX C

Action of the Enzyme on β -Carbonaphthoxycholine Iodide (78)

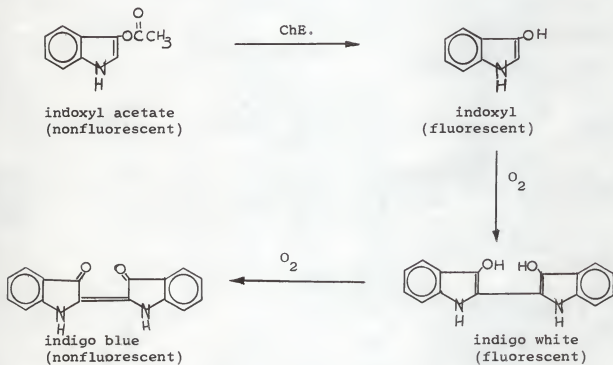
Upon cleavage of ester linkage between choline and carboxylic acid, β -naphthylcarbonic acid is released which spontaneously decarboxylates to yield β -naphthol. Diazo Blue B and β -naphthol couple to form a red, insoluble azo dye at the site of enzymatic activity.



APPENDIX D

Action of the Enzyme on Indoxyl Acetate (37)

Cholinesterase hydrolyzes the ester linkage of indoxyl acetate yielding the highly fluorescent indoxyl. In the presence of oxygen, indoxyl is oxidized to indigo white and then to indigo blue, a non-fluorescent compound. At pH's less than 7, the fluorescent material is stable.



APPENDIX E

Steady-State Stacking Regulating Function

Ornstein (63) modified Kohlrausch's Regulating Function (48) for application to disc electrophoresis as follows:

At pH's near the pKa, only part of the molecules of acids and bases will be ionized such that their average velocity will be $dx/dt = Vm_x$, where m is the mobility and x is the fraction of dissociation.

Consider two solutions layered over each other in a cylinder with the upper "solution 1" containing r and t ions and the lower "solution 2" containing s and t ions (t of opposite charge to r and s and s of greater mobility than r). In order for a steady-state boundary to be established and maintained between these two solutions upon application of a potential, their mobility-voltage gradient products must be equal. $dx/dt = V_1 m_r x_r = V_2 m_s x_s$

$$\text{From Ohm's Law } V = IR = I/\lambda A \quad \lambda = e \sum c_m z$$

where λ is electrical conductivity, e , the charge of the electron, c the ion concentration, z , the charge of the ion, and A , the area. From this relationship, a form of Kohlrausch's Regulating Function (48) is established.

$$\frac{m_r x_r}{\lambda_1} = \frac{m_s x_s}{\lambda_2}$$

$$\frac{m_r x_r}{\sum c_1 m_1 z_1} = \frac{m_s x_s}{\sum c_2 m_2 z_2}$$

This was expanded by Ornstein (63) for application to disc electrophoresis.

$$\frac{m_r x_r}{c_r m_r z_r + c_{t_1} m_t z_t} = \frac{m_s x_s}{c_s m_s z_s + c_{t_2} m_t z_t}$$

Electrical neutrality in each solution requires that $c_r z_r = -c_{t_1} z_t$ and $c_s z_s = -c_{t_2} z_t$.

$$\frac{m_r x_r}{c_r z_r (m_r - m_t)} = \frac{m_s x_s}{c_s z_s (m_s - m_t)}$$

Letting the total concentration of the molecular species (R) and (S) be c_r/x_r and c_s/x_s then

$$\frac{(R)}{(S)} = \frac{x_s c_r}{x_r c_s} = \frac{m_r z_s (m_s - m_t)}{m_s z_r (m_r - m_t)}$$

Therefore, this is the ratio of the total concentration of the molecular species necessary to set up equality between the mobility-voltage gradient products to establish and maintain a steady-state boundary which in disc electrophoresis results in concentration of protein.

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STUDIES ON PREPARATIVE POLYACRYLAMIDE
DISC ELECTROPHORESIS

by

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ABSTRACT

A staining procedure for protein bands in disc electrophoresis which does not require electrophoretic destaining was described. This procedure calls for 20 minutes gel immersion in 20% sulfosalicylic acid followed by about 2 hours staining in 0.25% Coomassie Brilliant Blue R250. Background dye was readily removed by a few washes in distilled water. The gels could be stored indefinitely in 7% acetic acid. This Coomassie Blue procedure proved to be a very sensitive and convenient method for staining fractionated protein bands and gave results comparable or better than with amido black staining.

The second part of this work described a model preparative disc electrophoresis procedure for initial purification of a component in a sample mixture. This method utilized the "steady-state stacking" phenomenon provided by Kohlrausch's "regulating function." The fractionation column consisted of a cellulose nitrate centrifuge tube with a 7% polyacrylamide gel plug, a 2% polyacrylamide gel stacking area, and a sample layer. The sample layer volumes used varied from 0.25 ml (13 mg protein) to 4.0 ml (344 mg protein) of horse serum. In every case, the protein was electrophoretically stacked in the 2% polyacrylamide stacking gel.

With two horse serum enzymes, cholinesterase (3.1.1.8) and carboxylesterase (3.1.1.1), as the model components, the effectiveness of separation in the stacked area was determined by freezing the gel after electrophoresis and assaying consecutive slices taken through the stacked area for protein and enzyme activity. The results showed a broad concentrated protein area but with two distinctly separated

esterases. Analytical fractionation and protein and enzyme staining of composite samples, obtained from pooled individual slices, also showed this esterase separation. Comparison of analytically fractionated commercial cholinesterase and composite No. 3 was made to show the potential of this preparative separation for component purification.

This work has demonstrated that steady-state stacking preparative disc electrophoresis can be successfully employed for a one-step purification of a single component from a large volume of complex mixture. This procedure could be scaled up to considerably larger volumes if the electrophoretic conditions were carefully controlled. In principle a homogeneous protein should be obtainable from a thin "stacked" section. Although homogeneous protein components were not obtained with the present method, it seems quite possible that one or two repeat runs through the procedure might achieve that result.